C-170 Evaluation of Dye Tolerance Media for the Identification of *Brucella* Species

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ABSTRACT

Brucella is a highly pathogenic bacterium that is classified as a potential agent of bioterrorism and should be handled in BSL-3 facilities. Presumptive identification is made by characteristic gram stain and colony morphology; catalase, oxidase and urease reactions; and *Brucella* antisera agglutination tests. However, identification of *Brucella* to the species level cannot be accomplished using routine biochemical tests. Although molecular techniques may unambiguously discriminate between *B. abortus, B. melitensis, and B. suis*, they are not readily available. Inhibition of growth by varying concentrations of thionin and basic fuchsin dyes is a useful method for speciation. This study evaluated sets of dye tolerance media obtained from Hardy Diagnostics, Santa Maria, CA. Thionin was tested at dilutions of 1:25K, 1:50K and 1:100K. Basic fuchsin was tested at dilutions of 1:50K and 1:100K. A heart infusion agar pour plate was used as a growth control. The plates were inoculated with a single streak of a 0.5 McFarland suspension of the organism, incubated at 35º C for up to 4 days, and examined daily for growth. Twenty-five isolates, identified by local county health departments as *B. melitensis* (19), *B. abortus* (3) and *B. suis* (3), were tested. An additional 18 isolates, identified by conventional biochemical methods as *Brucella* species only, were also tested. The speciation of the 25 known *Brucella* isolates by the dye method agreed 100 per cent with the reference identifications. The speciation of the additional *Brucella* isolates resulted in the identification of 13 as *B. melitensis* and 5 as *B. suis*. The Hardy dye tolerance test is a reliable and convenient method for speciation of the three most common *Brucella* pathogens.

INTRODUCTION

Brucella spp. are small Gram negative coccoid rods that can cause serious infections in humans and animals. Confirmation of *Brucella* species is important in the epidemiology of disease. Presumptive identification is based on the detection of fastidious Gram negative coccobacilli that are oxidase, catalase, and urea positive (4). Since the adoption of the Code of Federal Regulations 42 Part 73- The Use and Transfer of Select Agents and Toxins (CFR42 Part 73), only presumptive identification of *Brucella* can be performed in most clinical laboratories with confirmation and speciation being performed at level 2 sentinel laboratories (1). These laboratories may have the ability to perform molecular testing for identification of genus (2,5) but many still depend upon conventional methods for speciation including the dye tolerance test. In this study, we evaluated the Hardy Dye Tolerance media (Hardy Diagnostics, Santa Maria, CA) to identify the most common *Brucella* species (*B. melitensis, B. abortus*, and *B. suis*) (6,7).

METHODS AND MATERIALS

Strains: A total of 43 *Brucella* isolates were tested. Twenty-five isolates had been previously identified by molecular and conventional methods at local county health departments. Nineteen had been identified as *B. melitensis*, three as *B. abortus*, and three as *B. suis.* An additional 18 isolates that had been previously identified as *Brucella spp*. by conventional methods were also tested. Isolates were grown on Chocolate agar at 35-37°C in 5-7% CO_2 for 72 hours prior to testing.

Materials: Each isolate was inoculated to a set of dye tolerance media. Each set contained 6 tubes of prepared medium. Three tubes contained Heart Infusion Agar (HIA) with dilutions of Thionin (1:100,000, 1:50,000, and 1:25,000). Two tubes contained HIA with dilutions of Basic Fuschin (1:100,000 and 1:50,000). The growth control tube contained only Heart Infusion Agar. The tubes were liquefied by boiling and pour plates were prepared. The plates were allowed to cool and solidify at room temperature. All plates were used the day of preparation.

Methods: All procedures were performed according to the manufacturer's instructions (3). A suspension of a 72-hour culture was made in phosphate buffered saline equivalent to the density of a 0.5 McFarland standard. Each test plate was inoculated with a 10 µl loopful of the organism suspension inoculated in a single streak down the middle of the plate. Each set of plates was incubated in 5-7% CO_2 at 35-37°C for 96 hours. The plates were examined for growth each day. All work was performed in accordance with CFR42 Part 73 in a Bio-Safety Level 3 (BSL-3) laboratory.

Identification: Species identifications were made based on the following accepted schemes (Table 1).

Table 1: Interpretation of Results

	Thionin	Thionin	Thionin	Basic Fuschin	Basic Fuschin	HIA
Dye Concentration	1:25,000	1:50,000	1:100,000	1:50,000	1:100,000	Control
B. melitensis (biovar 1-3)	-	+	+	+	+	+
B. abortus biovar 1	-	-	-	+	+	+
2	-	-	-	-	-	+
3	+	+	+	+	+	+
4	-	-	-	+	+	+
5	-	+	+	+	+	+
6	-	+	+	+	+	+
7	-	+	+	+	+	+
B.suis biovar 1	+	+	+	-	-	+
2	-	+	+	-	-	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	Not Tested	-	-	+

RESULTS

Plates were observed daily for the first sign of growth on the control (HIA) plate. Final results were recorded on the fourth day by comparison of growth on the dye plates compared to growth on the control plate. The results were recorded on the data sheets.

There was 100% agreement for the 25 previously identified isolates (Table 2). Of the 18 *Brucella spp*. not previously speciated, 13 were identified as *B. melitensis* and 5 as *B. suis (Table 3).*

Six isolates had results that were read as weakly positive. Each of the isolates was re-tested to confirm the results. They are:

1. Isolate 12, *B. melitensis,* weakly positive on the Thionin 1:50,000 plate and positive on re-test. (Table 2)
2. Isolate 16, *B. melitensis,* weakly positive on the Thionin 1:25,000 plate and negative on re-test. (Table 2)
3. Isolate 21, *B. abortus*, weakly positive on the Thionin 1:25,000 and 1:50,000 plates and both were negative on re-test. (Table 2)
4. Isolate 24, *B. suis*, weakly positive on the Basic Fuschin 1:50,000 and 1:100,000 plates and both were negative on re-test. (Table 2)
5. Isolate 10, *Brucella spp.*, weakly positive on the Thionin 1:50,000 and Basic Fuschin 1:50,000 plates and both were positive on re-test. (Table 3)
   Isolate 11, *Brucella spp.*, weakly positive on the Basic Fuschin 1:50,000 plate and positive on re-test. A positive rapid urease (less the 5 minutes) was used to determine the identity of this isolate. (Table 3)
6. Isolate 11, *Brucella spp.*, weakly positive on the Basic Fuschin 1:50,000 plate and positive on re-test. A positive rapid urease (less the 5 minutes) was used to determine the identity of this isolate. (Table 3)

Table 2: Results of Testing on Known *Brucella* Isolates

NO	ORGANISM	RESULTS						IDENTIFICATION
		Thionin 1:25,000	Thionin 1:50,000	Thionin 1:100,000	Basic Fuchsin 1:50,000	Basic Fuchsin 1:100,000	HAI Cont.	
1	*B. melitensis* 17109	-	+	+	+	+	+	*B. melitensis*
2	*B. melitensis* 17110	-	+	+	+	+	+	*B. melitensis*
3	*B. melitensis* 28851	-	+	+	+	+	+	*B. melitensis*
4	*B. melitensis* 17111	-	+	+	+	+	+	*B. melitensis*
5	*B. melitensis* 17112	-	+	+	+	+	+	*B. melitensis*
6	*B. melitensis* 17113	-	+	+	+	+	+	*B. melitensis*
7	*B. melitensis* 17114	-	+	+	+	+	+	*B. melitensis*
8	*B. melitensis* 17115	-	+	+	+	+	+	*B. melitensis*
9	*B. melitensis* 17117	-	+	+	+	+	+	*B. melitensis*
10	*B. melitensis* 17121	-	+	+	+	+	+	*B. melitensis*
11	*B. melitensis* 17122	-	+	+	+	+	+	*B. melitensis*
12	*B. melitensis* 17123	-	+w (+)	+	+	+	+	*B. melitensis*
13	*B. melitensis* 17127	-	+	+	+	+	+	*B. melitensis*
14	*B. melitensis* 25076	-	+	+	+	+	+	*B. melitensis*
15	*B. melitensis* 25077	-	+	+	+	+	+	*B. melitensis*
16	*B. melitensis* 25078	+w (-)	+	+	+	+	+	*B. melitensis*
17	*B. melitensis* 25079	-	+	+	+	+	+	*B. melitensis*
18	*B. melitensis* 25080	-	+	+	+	+	+	*B. melitensis*
19	*B. melitensis* 56777	-	+	+	+	+	+	*B. melitensis*
20	*B. abortus* 23267	-	-	+	+	+	+	*B. abortus*
21	*B. abortus* 17126	-	+w (-)	+w (-)	+	+	+	*B. abortus*
22	*B. abortus* 75177	-	-	-	+	+	+	*B. abortus*
23	*B. suis* 28850	+	+	+	-	-	+	*B. suis*
24	*B. suis* 17125	+	+	+	+w (-)	+w (-)	+	*B. suis*
25	*B. suis* 8681	+	+	+	-	-	+	*B. suis*

Table 3: Results of *Brucella spp*. Not Previously Identified

NO.	ORGANISM	RESULTS						IDENTIFICATION
		Thionin 1:25,000	Thionin 1:50,000	Thionin 1:100,000	Basic Fuschin 1:50,000	Basic Fuschin 1:100,000	HAI Cont.	
1	*Brucella spp.* 17116	-	+	+	-	-	+	*B. suis*
2	*Brucella spp.* 17118	+	+	+	-	-	+	*B. suis*
3	*Brucella spp.* 17119	-	+	+	+	+	+	*B. melitensis*
4	*Brucella spp.* 17124	-	+	+	+	+	+	*B. melitensis*
5	*Brucella spp.* 17128	+	+	+	-	-	+	*B. suis*
6	*Brucella spp.* 17129	-	+	+	+	+	+	*B. melitensis*
7	*Brucella spp.* 17130	-	+	+	+	+	+	*B. melitensis*
8	*Brucella spp.* 17131	-	+	+	+	+	+	*B. melitensis*
9	*Brucella spp.* 17132	-	+	+	+	+	+	*B. melitensis*
10	*Brucella spp.* 17133	-	+w (+)	+	+w (+)	+	+	*B. melitensis*
11	*Brucella spp.* 17134	+	+	+	+w (+)	+	+	*B. suis*
12	*Brucella spp.* 19309	-	+	+	+	+	+	*B. melitensis*
13	*Brucella spp.* 19621	-	+	+	+	+	+	*B. melitensis*
14	*Brucella spp.* 19974	+	+	+	-	-	+	*B. suis*
15	*Brucella spp.* 23266	-	+	+	+	+	+	*B. melitensis*
16	*Brucella spp.* 23268	-	+	+	+	+	+	*B. melitensis*
17	*Brucella spp.* 25075	-	+	+	+	+	+	*B. melitensis*
18	*Brucella spp.* 25503	-	+	+	+	+	+	*B. melitensis*

Fig. 1 *B. mel.* on HAI (Control)

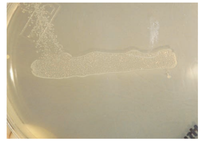


Fig. 2 *B. mel.* on Basic Fucshin

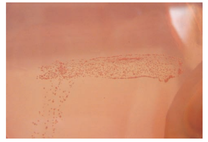


Fig. 3 *B. mel.* on Basic Fucshin

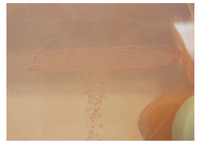
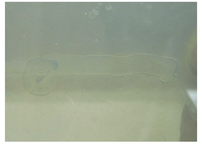


Fig. 4 *B. mel.* on Thionin 1:25,000 (No Growth)

Fig. 5 *B. mel.* on Thionin 1:50,000

Fig. 6 *B. mel.* On Thionin 1:100,000



DISCUSSION

Dye tolerance media are widely recognized as a means of identification of *Brucella* species. Using the Hardy media was easy and did not require extensive preparation. There was sufficient growth to read many of the isolates at 3 days but for the all isolates were evaluated at 4 days as per the protocol. Growth on the plates was clearly visible and the reactions were easy to read. Care must be taken to insure an organism suspension equivalent to the density of a 0.5 McFarland standard. Weakly positive growth was observed on plates for six isolates. On re-test, three had positive growth and three were negative for growth. One identification (*B. suis*) was based on a rapid positive urease (<5 min.) to differentiate it from strains of *B. abortus* that have similar dye tolerance patterns. Because the dye is in the medium, any inoculum on the plate tends to pick up the dye. Care must be taken when examining the plates. The plates have to be read in comparison to the control plate. Reading discoloration may cause false-positive results but true growth must be detected in order to call a result positive.

CONCLUSION

The Hardy Dye Tolerance test is a reliable method for the identification of *Brucella spp*. The media are easy to store, prepare, and can be used by laboratories to speciate *Brucella spp.* where molecular methods are not available.

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